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Liquid chromatographic/mass spectrometric determination of biologically active alkaloids in extracts of *Peschiera fuschiaefolia*

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Four alkaloids were isolated from an alcoholic extract of the bark from the stem of *Peschiera fuschiaefolia*. Two of these compounds, voacamine and voacamidine, are dimeric alkaloids which are thought to be responsible for the antimalarial activity of these extracts. The mass spectra and the response factors of these four compounds were obtained by liquid chromatography/mass spectrometry in the electrospray positive ionization mode. The concentrations of these alkaloids were measured in two different *P. fuschiaefolia* extracts. The ion chromatograms of the two extracts were compared on the basis of their $[M + H]^+$ and $[M + 2H]^{+2}$ ions characteristic of various alkaloids previously isolated from *P. fuschiaefolia* bark. The two extracts were found to differ mostly in the relative concentrations of the dimeric alkaloids. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography/mass spectrometry; alkaloids; voacamine; malaria; plant extracts

INTRODUCTION

Peschiera fuschiaefolia is an infesting plant that grows in South America. This plant belongs to the subfamily of Tabernaemontanoideae of the Apocynaceae. It is a small tree that infests pastures in Brazil and which has to be kept under control with pesticides.¹ Various families of alkaloids have been isolated from many Apocynaceae, such as those related to vobasine, affinisine, voacangine and voacamine^{2–4} (Fig. 1). The last compound and its analogs are dimeric alkaloids.

Recently, Federici *et al.*⁵ reported that *P. fuschiaefolia* stem and root bark extracts have *in vitro* activity against *Plasmodium falciparum*, the parasite responsible for malaria. Among the various alkaloids isolated from that plant, they found that voacamine has the highest *in vitro* activity against a normal (W₂) and a chloroquine-resistant strain (D₆). They also found that crude stem and root bark extracts have good *in vitro* activities, the latter being even more active than voacamine itself. The higher activity of the root bark extract was attributed to the larger amounts of dimeric alkaloids, which were much more active *in vitro* than the monomeric compounds.

Bertelli and Rossi⁶ showed that crude stem bark extract has good antimalarial activity in humans, and industrial

production of extracts from the bark of the stem has already started. One of the problems with crude extracts is the variations in the concentration of the active ingredients from batch to batch. Because dimeric alkaloids such as voacamine are thought to be responsible for the biological activity of the extract, a simple and rapid analytical technique was needed to quantitate voacamine in these extracts. Because of the complexity of these mixtures, which include a large number of closely related alkaloids, chromatographic separation must be performed. Considering that the high molecular mass of these compounds would not be compatible with gas chromatography, a liquid chromatographic/mass spectrometric (LC/MS) method was developed. Because pure voacamine is not available commercially, it had to be isolated from the extract to determine its response factor. We were also interested in comparing the extracts of two different batches of *P. fuschiaefolia* bark to look for the variations from batch to batch of the concentration of voacamine and of other dimeric and monomeric alkaloids.

EXPERIMENTAL

Plant extracts

Two *P. fuschiaefolia* stem bark extracts were provided by Millenia Hope (Montreal, Que, Canada). These extracts were obtained by treatment of the dried bark with ethanol. The two extracts were obtained from two different batches of bark, collected a few months apart. The first extract was a gum and the second was obtained as a powder.

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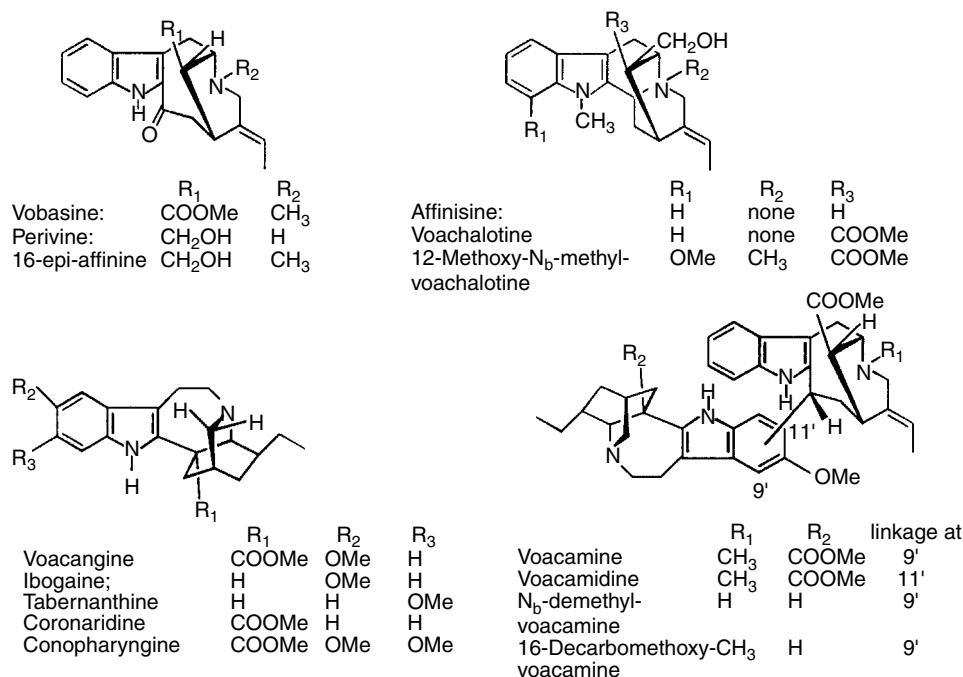


Figure 1. Structures of alkaloids isolated from *P. fuschiaefolia* bark according to Federici *et al.*⁵

Chemicals

All the solvents were from EM Science (VWR, Mont-Royal, Que, Canada) and were of Omnisolv grade. Silica gel (230–400 mesh) was obtained from Silicycle (Quebec, Que, Canada). Preparative (1 mm) PK6F (60 Å) thick-layer chromatographic (TLC plates) were supplied by Whatman (Clifton, NJ, USA).

Equipment

The counter current distribution (CCD) instrument was a Model 624 high-speed counter current chromatograph from Pc (Potomac, MD, USA). The LC/MS system was composed of an HP HPLC 1100 system (Agilent Technologies, Pointe-Claire, Que) and a Micromass Quattro II triple-quadrupole mass spectrometer (Micromass Canada, Pointe-Claire, Que, Canada). The HPLC system was equipped with a Zorbax Eclipse XDB (Chromatographic Specialties, Brockville, Ont, Canada) 5 µm C₈ reversed-phase column (150 × 4 mm i.d.). The effluent from the column was split to 10% using a Valco Tee before entering into the mass spectrometer through a Z-spray interface (Micromass).

Isolation of the alkaloids

Isolation of the alkaloids was performed with the gum which contained 12.8% of residual water. The powder did not contain any residual water. All the yields reported in this study were converted in terms of dry mass of the initial extract.

The extract (100 g) was dissolved in 1 l of Milli-Q water, the pH adjusted to 9 with Na₂CO₃ and the solution extracted three times with 250 ml of dichloromethane to give 8.8 g of an oil. This residue (20 g) was dissolved in 200 ml of dichloromethane and extracted three times with 150 ml of an aqueous citric acid–Na₂HPO₄ buffer (14.0 g l⁻¹ of Na₂HPO₄ and 10.0 g l⁻¹ of citric acid in of water) at pH 5. The aqueous

fractions were discarded and the organic layer was extracted three times with 150 ml of an aqueous citric acid solution (3.1 g l⁻¹) at pH 3.5. The aqueous fractions were combined, the pH was adjusted to 9.0 with Na₂CO₃ and the solution was extracted three times with 150 ml of dichloromethane to give 3.4 g of an oil.

This residue was then purified by CCD between dichloromethane and phosphate buffer. A 1 g amount of residue dissolved in 3 ml of dichloromethane was introduced into the instrument. The rotation speed was 250 rpm and the flow-rate of the phosphate buffer was 2 ml min⁻¹. The pH of the buffered phosphate solution was decreased stepwise according to the following scheme: 310 ml at pH 5.0, 40 ml at pH 4.0, 430 ml at pH 3.5 and finally 310 ml at pH 2.1. Fractions at pH 4.0 and 3.5 were combined, the pH was adjusted at 9.0 with Na₂CO₃ and the solution was extracted three times with 50 ml of dichloromethane to give 250–300 mg of a mixture of alkaloids.

This alkaloid mixture (500–600 mg) in a minimum volume of dichloromethane–methanol (95:5) was then applied on a 40 × 4.7 cm i.d. flash silica column. The alkaloids were eluted using a methanol–dichloromethane gradient. Typically, 600 mg of the initial mixture gave 200–300 mg of semi-purified alkaloids.

These compounds were then deposited on preparative thick-layer chromatographic plates. Typically 100 mg were deposited per plate and eluted with a mixture of 10% methanol in dichloromethane. The alkaloids were visualized by UV irradiation and the bands scraped off the plates and eluted with 40% methanol in dichloromethane. Typically, 100 mg of semi-purified voacamine gave 40 mg of a mixture of two voacamine isomers and 40 mg of other alkaloids. The two voacamine isomers were purified using another preparative TLC plate using ethyl acetate–chloroform–methanol (40:50:10). Typically, 100 mg of a mixture of the two isomers

gave 40 mg of voacamine ($R_f = 0.54$) and 30 mg of the other isomer, namely voacamidine (Fig. 1) ($R_f = 0.48$). The other two alkaloids, vobasine ($R_f = 0.44$) and voachalotine ($R_f = 0.61$) (Fig. 1) were repurified separately on preparative TLC plates using the same eluent.

Analytical parameters

The analyses were performed by LC/MS using a water–acetonitrile gradient. Phase A contained 0.1% TFA in water while phase B contained 10% water in acetonitrile and 0.1% TFA. The gradient is shown in Table 1. The flow-rate was $400 \mu\text{l min}^{-1}$ and the volume injected was $50 \mu\text{l}$.

The mass spectrometer was operated in the positive electrospray ionization mode. The instrument parameters were as follows: capillary voltage, 3.2 kV; cone voltage, 32 V; extractor, 5 V; nebulizing gas flow-rate, 15 l min^{-1} ; drying gas flow-rate, 50 l min^{-1} ; source temperature, 120°C ; and desolvation gas temperature, 150°C . The scanning mass range was 130–750 Da. MS/MS experiments were performed with argon at 2×10^{-3} mbar. Analysis of the purified compounds was performed in the continuum mode and quantification in the centroid mode.

Calibration curves

The purified alkaloids were individually dissolved in a known amount of dichloromethane. A precise volume of the solution was taken, evaporated and diluted in acetonitrile–water (18:72) containing 0.1% TFA. Final concentrations were 100, 75, 50 and 25 mg l^{-1} . The samples were analysed by LC/MS and the response factor was determined using ions characteristic of each compound. These ions appeared nominally at m/z 353 ($[\text{M} + 2\text{H}]^{2+}$) for voacamine and voacamidine, m/z 353 ($[\text{M} + \text{H}]^+$) for vobasine and m/z 367 ($[\text{M} + \text{H}]^+$) for voachalotine. A calibration curve was constructed for each of these ions using solutions of each concentration.

Determination of alkaloids in the extract

The extract (300 mg) was dissolved in 50 ml of 2% aqueous acetic acid solution, the pH adjusted to 9.0 with Na_2CO_3 and the solution extracted three times with 30 ml of dichloromethane. The organic fractions were combined, dried with anhydrous Na_2SO_4 , filtered and evaporated on a rotary evaporator. The residue was then diluted with dichloromethane in a 25 ml volumetric flask. A $500 \mu\text{l}$ aliquot was taken, evaporated to dryness under nitrogen and the residue dissolved in $500 \mu\text{l}$ of acetonitrile–water (18:72) containing 0.1% TFA. The solution was sonicated for 5 min,

passed through a $0.2 \mu\text{m}$ PTFE filter and $50 \mu\text{l}$ of the solution were injected. The analyses were performed in triplicate with the gum.

To measure the recovery of the alkaloids throughout sample preparation, incremental amounts of each of the purified alkaloids were added to samples of the gum prior to their extraction and analysis. To ensure that the purified alkaloids would be incorporated properly into the viscous matrix, 1.00 g of the extract was dissolved in 50 ml of ethanol prior to addition of the standards in dichloromethane. The solvent was then evaporated and the sample extracted as described previously.

RESULTS AND DISCUSSION

Four alkaloids were purified from the gum. These compounds were present in the fractions containing voacamine obtained after CCD purification. They were separated from voacamine by TLC. Voacamidine, being an isomer of voacamine, had a TLC R_f value very similar to that of voacamine but could be separated from it. Interestingly, voacamidine had only been isolated from *P. fuschiaefolia* root bark and not the stem bark of that plant.⁵

Confirmation of the structure of the various alkaloids isolated was performed using ^{13}C and ^1H NMR and mass spectrometry. Their ^{13}C and ^1H NMR spectra were identical with those described in the literature.^{2–4} The mass spectra of these four compounds were obtained in the positive electrospray ionization mode. The mass spectrum of voacamine (molecular mass (M_r) 704 Da) presents $[\text{M} + \text{H}]^+$ ions at m/z 705.1, doubly charged ions at m/z 394.3 ($[\text{M} + 2\text{H} + 2\text{CH}_3\text{CN}]^{2+}$), doubly charged ions at m/z 373.8 ($[\text{M} + 2\text{H} + \text{CH}_3\text{CN}]^{2+}$), doubly charged ions at m/z 353.3 ($[\text{M} + 2\text{H}]^{2+}$) and doubly charged ions at m/z 337.6 ($[\text{M} + 2\text{H} - \text{OCH}_3]^{2+}$) [Fig. 2(a)]. This latter mode of fragmentation was confirmed by the fragment ion spectra of the m/z 353.3 and 705.1 ions. They present m/z 337.6 and 674 ions, respectively, corresponding to loss a methoxy group from the parent ions (results not shown). The mass spectrum of voacamidine is almost identical with that of voacamine [Fig. 2(b)]. The mass spectrum of vobasine ($M_r = 352$ Da) presents $[\text{M} + \text{H}]^+$ ions at m/z 353.0 and ions at m/z 394.2 ($[\text{M} + \text{H} + \text{CH}_3\text{CN}]^+$) [Fig. 2(c)]. The mass spectrum of voachalotine ($M_r = 366$ Da) presents $[\text{M} + \text{H}]^+$ ions at m/z 367.1 and ions at m/z 408.1 ($[\text{M} + \text{H} + \text{CH}_3\text{CN}]^+$) [Fig. 2(d)]. The low-abundance ions at m/z 705.0 and 733.0 in the spectra of vobasine and voachalotine [Fig. 2(c) and (d)] are dimeric $[2\text{M} + \text{H}]^+$ ions. The HPLC retention times of vobasine (14.5 min) and voachalotine (19.7 min) are very different from that of voacamine (27.4 min) but voacamidine elutes at 26.8 min, very close to voacamine itself. Because both compounds present almost identical mass spectra, their identification during the analysis of the crude extract will rely on their slightly different retention times.

The calibration curves are presented in Figure 3. To improve the accuracy, the most abundant ions of the spectrum of each compound were selected to construct the calibration curves. These ions were the $[\text{M} + \text{H}]^+$ ions of the monomeric alkaloids and the $[\text{M} + 2\text{H}]^{2+}$ ions of the

Table 1. LC gradient used for alkaloid separation

Time (min)	A (%)	B (%)
1.0	80	20
5.0	70	30
30.0	50	50
37.0	0	100
39.0	0	100

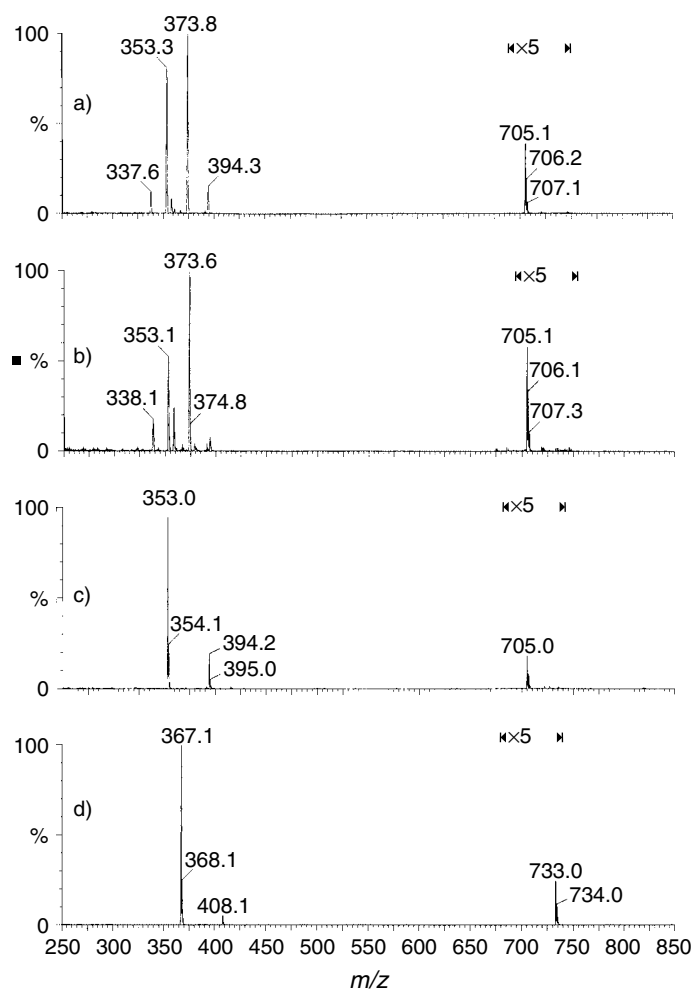


Figure 2. Electro spray positive mass spectra of purified (a) voacamine, (b) voacamidine, (c) vobasine and (d) voachalotine.

dimeric alkaloids [Fig. 2(a) and (b)]. Varying the voltage on the electrospray probe or on the entrance cone did not increase significantly the abundance of the $[M + H]^+$ ions of voacamine and voacamidine compared with those of their $[M + 2H]^{2+}$ ions. These calibration curves showed good linearity with a correlation coefficient (r^2) > 0.98 in all cases.

The total ion chromatogram (TIC) of the gum is presented in Fig. 4(a) and shows that a large number of peaks are present. The four alkaloids were quantitated in the two extracts. The gum was analysed in triplicate to verify the reproducibility of the method, while the powder was analysed once. The results are presented in Table 2. The reproducibility of the method is good, as shown by the standard deviations obtained for the triplicate analyses with the gum. The percentage recoveries with spiked standards were also excellent, indicating that the extraction of these alkaloids by this procedure was quantitative.

Table 2 also shows that voacamine, voacamidine and voachalotine in the powder are present in much greater amounts than in the gum. Figure 4(b) presents the TIC of the powder. These two profiles differ considerably, especially in the later eluting portion of the chromatogram, where voacamine and other dimeric alkaloids elute. Table 3 gives the relative abundances of ions characteristic of alkaloids known to be present in the stem bark of *P. fuschiaefolia* compared with the relative amounts of these alkaloids

actually isolated from the plant.⁵ Because these compounds are not commercially available in the pure state, one cannot positively identify these compounds in these extracts on the sole basis of their $[M + H]^+$ ions if they are observed at more than one retention time. Some other ambiguities also arise from the fact that some alkaloids are isomers of each other, such as ibogaine and tabernanthine, or isobaric, such as perivine and coronaridine. For the first two compounds, which have not been reported in *P. fuschiaefolia*, the observed m/z 311.1 ions were attributed to either one of these two compounds, whereas for perivine and coronaridine, assignment was made on the basis of that coronaridine was isolated in much larger amounts than perivine from stem bark.⁵

The relative proportions of the various alkaloids listed in Table 3 differ from those obtained from the isolated yields. Most notably, conopharyngine, which represented 43% of all the isolated alkaloids listed below, only represented 1.8% and 0.8% of the ions attributed to the alkaloids in the gum and in the powder, respectively. This could be due either to differences in the composition of the bark or to losses of the other, less abundant, compounds in the isolation process itself.

The spectrum of vobasine shows ions at m/z 705.1 and 353.0 that also appear in the spectrum of voacamine and voacamidine. The former correspond to $[2M + H]^+$

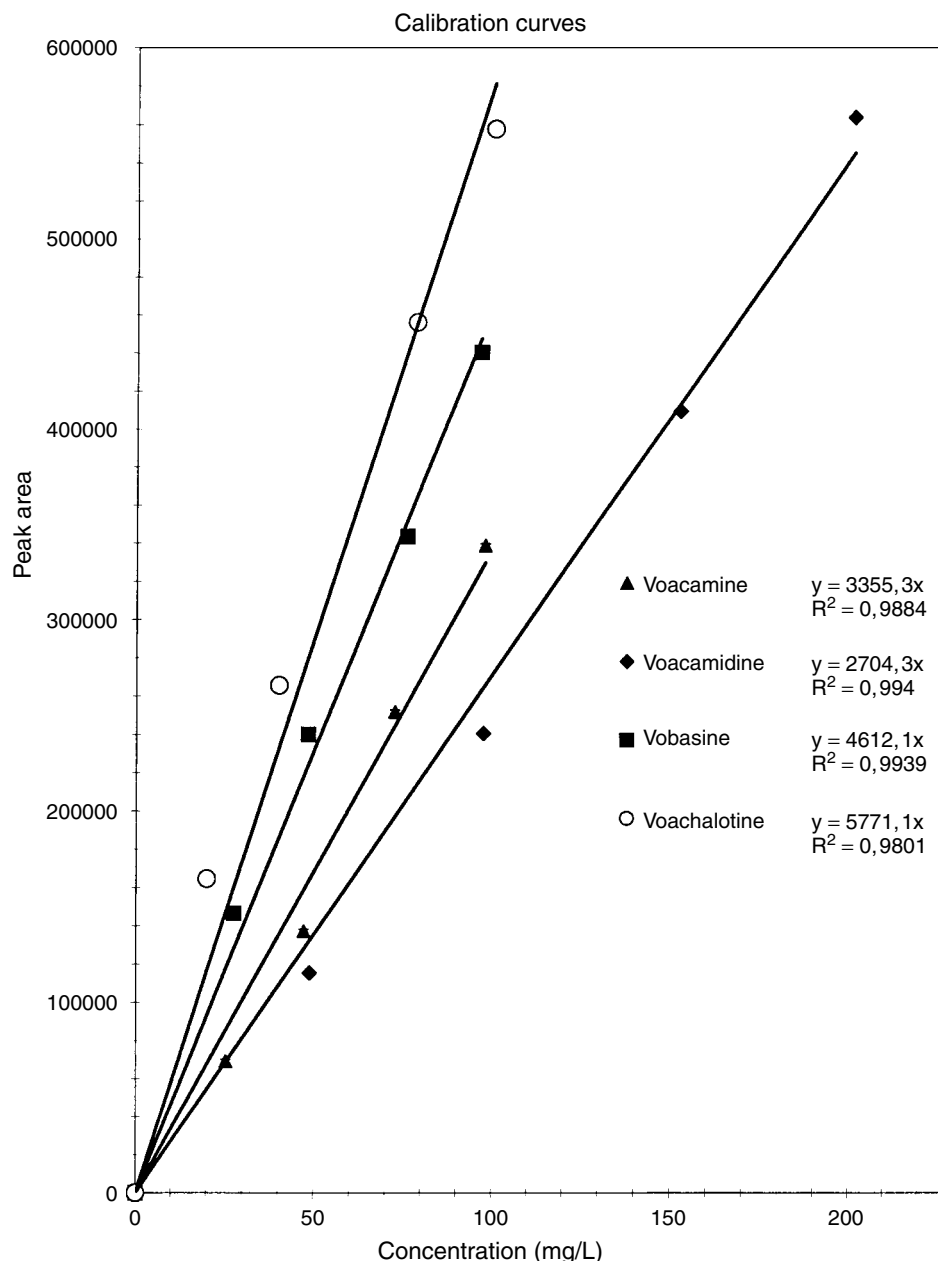


Figure 3. Dose–response curves of purified voacamine, voacamidine, vobasine and voachalotine.

ions, which could lead erroneously to assigning a dimeric structure to vobasine, while this is only an artefact of the ionization process. The $[M + H]^+$ ion of vobasine occurs at m/z 353.1, close to the $[M + 2H]^{2+}$ ion of voacamine (m/z 353.5) which could also give rise to ambiguities. However, the ^{13}C isotope peak of vobasine and voacamine occurs at m/z 354.1. At the resolution used, the $[M + H]^+$ ion peak of vobasine presents a clearly separated isotopic ion peak whereas for voacamine and voacamidine, their isotope peak is not resolved, leading to a broader peak. Thus analysis of the shape of the peak occurring nominally at m/z 353 reveals whether these are $[2M + H]^+$ or $[M + 2H]^{2+}$ ions and if the alkaloid is dimeric or not. In addition to that, the retention times of the higher molecular weight dimeric alkaloids are longer than those of the monomeric alkaloids.

Because all the dimeric alkaloids reported in this plant contain a moiety derived from vobasine, MS/MS

experiments were performed to see if these dimeric compounds produce ions that could be attributed to this vobasine sub-unit. The tandem mass spectra of the $[M + 2H]^{2+}$ ions of voacamine and voacamidine contain an abundant ion at m/z 180.1. A mechanism consistent with the formation of this ion is presented in Fig. 5. Indirect confirmation of the structure of the m/z 180.1 ions from vobasine was obtained by examining the tandem mass spectrum of the m/z 325.1 ions assigned to 16-epi-affinine in the chromatogram of the powder extract. 16-epi-affinine is an analog of vobasine in which the ester group is replaced by an alcohol (Fig. 1). The tandem mass spectrum of the $[M + H]^+$ ions of 16-epi-affinine show abundant ions at m/z 152.0, corresponding to the structure depicted in Fig. 5 and thus indirectly confirming the structure proposed for the m/z 180.1 ions of vobasine and the dimeric alkaloids derived from it. The mechanism depicted in Fig. 5 was further tested by deuterium labelling

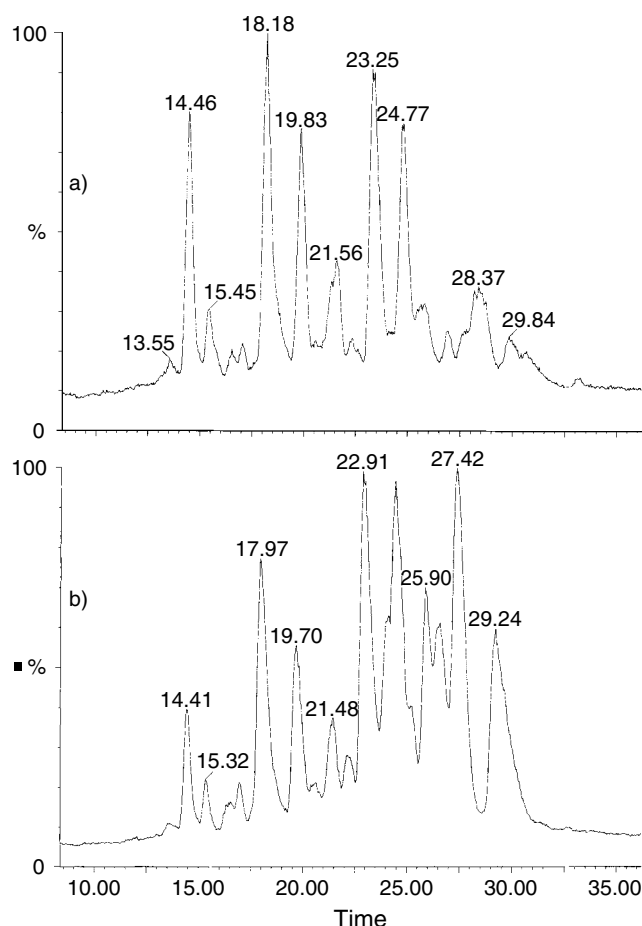


Figure 4. Total ion chromatogram (TIC) of (a) gum and (b) powder extract of *P. fuschiaefolia* stem bark.

Table 2. Concentrations (mg g^{-1} dry mass) of alkaloids in *P. fuschiaefolia* extract

Alkaloid	Gum (mg g^{-1})	Recovery (%) ^b	Powder (mg g^{-1})
Voacamine	1.70 (0.09) ^a	106	7.79
Vobasine	1.15 (0.03)	98	1.20
Voachalotine	2.85 (0.08)	97	5.57
Voacamidine	0.76 (0.06)	104	2.31

^a Numbers in parentheses are the standard deviations for triplicate measurements.

^b Measured in the gum.

experiments. Vobasine was dissolved in deuterium oxide containing traces of sulfuric acid and the $[\text{M} + \text{H}]^+$ ions, now occurring at m/z 355.0, were analysed in fragment ion mode. The fragment ions obtained were the same as in the non-labelled experiment, but were all shifted at higher values by 1 Da except for the m/z 180.1 ions. According to Fig. 5, the fragment at m/z 180.1 is an alkylated pyridinium ion and should not display deuterium enrichment after fragmentation of the deuterated parent compound. Furthermore, when a high voltage (100 V) was applied to the entrance cone in order to fragment the $[\text{M} + \text{H}]^+$ ions of unlabelled vobasine to produce the m/z 180.1 ions, MS/MS experiments on these

ions produced fragments at m/z 178.1, 165.1 and 162.0. These ions correspond to the loss of H_2 , CH_3 and CO , in agreement with the structure proposed in Fig. 5.

Tandem mass spectra were acquired for the $[\text{M} + 2\text{H}]^{2+}$ ions attributed to the dimeric alkaloids *N*₆-demethylvoacamine and 16-decarbomethoxyvoacamine and both showed ions at m/z 180, which confirms that these compounds contain a moiety derived from vobasine. In addition to the four dimeric alkaloids described in Table 3, three late-eluting compounds with $[\text{M} + \text{H}]^+$ ions at m/z 633.2, 733.2 and 747.1 were detected. These compounds all presented abundant $[\text{M} + 2\text{H} + \text{CH}_3\text{CN}]^{2+}$ and $[\text{M} + 2\text{H}]^{2+}$ ions. These ions all show, after MS/MS, fragments at m/z 180, suggesting that these compounds are unknown dimeric alkaloids, probably containing a moiety derived from vobasine.

From Tables 2 and 3 and Fig. 4, it can be concluded that there are considerable differences between the two extracts, the gum being enriched with compounds having shorter retention times such as 16-epi-affinine and 12-methoxy-4-methylvoachalotine, while the powder contains

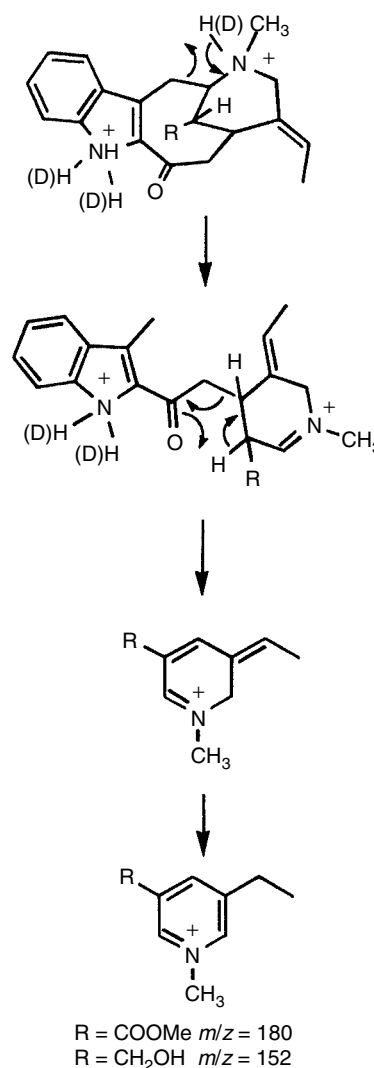


Figure 5. Proposed mechanism of formation of the m/z 180.1 and 152.0 ions obtained by MS/MS of the $[\text{M} + \text{H}]^+$ ions of vobasine and 16-epi-affinine.

Table 3. Relative abundances of the ions monitored in the gum and in the powder in comparison with the relative amounts isolated from the bark of *P. fuscaefolia*

Alkaloid	Ions monitored ^a	Retention time (min)	Relative amount isolated from the bark ^b	Relative abundance of the ions in the gum ^c (%)	Relative abundance of the ions in the powder ^c (%)
16-Epi-affinine	325	14.5	15.7	16.23	4.73
Vobasine	353	15.3	0.4	2.29	1.53
Affinisine	309	18.0	9.1	22.52	14.59
Conopharyngine	399	18.7	42.9	1.83	0.80
Voachalotine	367	19.7	0.9	6.26	10.14
Ibogaine or Tabernanthine	311	21.5	NR ^d	0.68	3.46
Voacangine	369	23.0	10.5	21.76	16.95
12-Methoxy-N _b - methylvoachalotine	411	24.5	NR	12.28	3.70
Perivine	339	ND ^e	0.9	ND	ND
Coronaridine	339	24.5	18.1	9.34	13.60
N _b -Demethylvoacamine	346 ^f	26.0	0.4	0.60	2.18
16-Decarbo- methoxyvoacamine	324 ^f	26.4	NR ^e	0.60	3.75
Unknown	317 ^f	25.2	NR	NM	0.53
Voacamidine	353 ^f	26.8	NR	0.68	1.75
Unknown	367 ^f	27.3	NR	NM	1.01
Voacamine	353 ^f	27.4	0.9	3.4	10.83
Unknown	374 ^f	29.2	NR	1.5	10.38

^a Ions monitored (nominal mass) were the $[M + H]^+$ ions except when mentioned otherwise.

^b Calculated as the relative concentration in the bark (in %) divided by the total concentration of all the alkaloids listed, according to Federici *et al.*⁵

^c Calculated as the abundance of the $[M + H]^+$ or $[M + 2H]^{2+}$ ions divided by the sum of all the corresponding alkaloid ions, in %.

^d Not reported.

^e Not detected.

^f The ions monitored for the dimeric alkaloids were the $[M + 2H]^{2+}$ ions.

much higher concentrations of the biologically active dimeric alkaloids, such as voacamine and voacamidine, 16-decarboxymethoxyvoacamine and N_b-demethylvoacamine. The proportion of the unknown dimeric alkaloids is also considerably larger in the powder than in the gum. Most notably, the $[M + 2H]^{2+}$ ions at m/z 374.1 of one of the unknown dimeric alkaloids is almost as abundant as the $[M + 2H]^{2+}$ ions of voacamine in the powder (Table 3).

CONCLUSION

A method was developed that allows the rapid quantification of voacamine and three other alkaloids in extracts of *P. fuscaefolia*. This method was simple and reproducible. The profiles of two different extracts were compared and one of them was found to contain higher concentrations of voacamine and voacamidine. Ions corresponding to two other dimeric alkaloids 16-decarbomethoxyvoacamine and

N_b-demethylvoacamine were also found in much higher concentrations in the powder extract. Three other ions corresponding to unknown but related alkaloids were also found in these extracts, mostly in the powder. The differences between the two extracts clearly show the variability in the alkaloids content of this plant-derived material, confirming the need for an analytical technique to determine these compounds.

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